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Original article

# Synthesis, hypoxia-selective cytotoxicity of new 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives

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ABSTRACT

We reported the synthesis, hypoxic cytotoxic activities and selectivities of 18 new 3-(alkoxymethylamino)-1,2,4-benzotriazine 1,4-dioxides. The synthesized compounds were screened *in vitro* against 5 cell lines: K562, SMMC-7721, A549, PC-3 and KB in hypoxia and in normoxia. Some of them showed higher or similar cytotoxic activity when compared to tirapazamine. Physico-chemical study showed the positive correlation between hypoxic activity and lipophilicity within a certain range. Preliminary mechanism study on the potent derivatives **4b**, **4l** and **4m** indicated that the cytotoxic activities of these compounds might be mediated by inducing apoptosis.



## 1. Introduction

Hypoxia, formed by chaotic growth and an inefficient microvasculature system within the tumor [1], not only drives tumor metabolism, progression, invasion, and metastasis [2–6], but also plays a negative role in the treatment of human tumors because of their resistance to radiotherapy and chemotherapy through various mechanisms [7–10]. Thus, tumor hypoxia gives an opportunity for the development of bioreductive agents, which can be selectively activated within hypoxic tissues [11–13].

A range of chemical classes has been explored since this concept was first proposed: N-oxides, quinones, nitro-aromatics, and metal complexes [14]. Tirapazamine (TPZ), a benzotriazine 1,4-dioxide, is one of such tumor-selective prodrug which undergoes enzymic one-electron reduction to form a DNA damaging species, which may affect DNA strand breaks [15–17]. TPZ has been studied extensively *in vitro* and *in vivo* and has already demonstrated significant activity in a wide range of clinical trials in combination with radiotherapies and cisplatin-based chemotherapies [18–20].

Physico-chemical property, associating closely with the penetration of anticancer drugs [21], is one of the primary concerns for molecules that targeting the hypoxic fraction of a solid tumor for hypoxic regions are far away from blood vasculatures. Although being a potent hypoxic antitumor compound, the ability of TPZ to kill hypoxic cells in tumors is generally limited by its poor extravascular transport [22,23]. Moreover, TPZ displays significant toxicities in the clinical setting [18,19]. Therefore, many efforts are currently involved in developing new analogues with higher biological activity and/or lower systemic toxicity. Hay's group had made a lot of contribution in this field. They applied the spatially resolved pharmacokinetic/pharmacodynamic (PK/PD) model to guide drug synthesis and identified several benzotriazine 1,4-dioxides with improved extravascular transport (EVT) and hypoxic activity [24,25].

In the previous work of our team, some lipophilic groups were introduced into the C-3 amino group to improve the activity of benzotriazine 1,4-dioxides. Most of the compounds showed more potent activity than TPZ [26,27]. The structure–activity relationships demonstrated that when the compound had an electron-donating substituent such as methoxy group on the 7-position of benzotriazine ring, it showed higher hypoxic selectivity than that of an electron-withdrawing substituent [27]. In order to find new molecules possessing cytotoxic activity and hypoxic selectivity, a series of 3-(alkoxymethylamino)-1,2,4-benzotriazine 1,4-dioxides were synthesized and evaluated for their cytotoxicity and selectivity *in vitro*.

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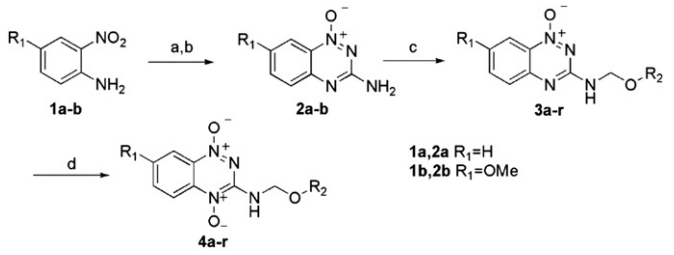



**Scheme 1.** The synthetic route of compounds **4a**–**r**. Reagents and conditions: (a) $NH_2CN$, cHCl, 100 °C; (b) 30% aq. NaOH, 100 °C; (c) $R_2OH$, $(HCHO)_n$, $CH_3COOH$, 85 °C; (d) MCPBA, $NaHCO_3$, DCM, 20 °C.

## 2. Results and discussion

### 2.1. Chemistry

The synthetic route of benzotriazine 1,4-dioxide analogues **4a**–**r** is shown in Scheme 1. Substituted nitroanilines **1a**–**b** were converted to 3-amino-1,2,4-benzotriazine 1-oxides **2a**–**b** using the method of Mason and Tennant [28]. Treatment of **2a**–**b** with paraformaldehyde and alcohol derivatives in the presence of catalytic amount of acetic acid at 85 °C for 3 h resulted in 3-(alkoxymethylamino)-1,2,4 -benzotriazine 1-oxides **3a**–**r** in high yields (70–90%). Oxidation of **3a**–**r** with MCPBA at room temperature in dichloromethane gave target compounds **4a**–**r**. The structures of all the newly synthesized compounds were determined by IR, $^1$H NMR, $^{13}$C NMR and mass spectrum.

### 2.2. Pharmacology

#### 2.2.1. In vitro cytotoxic activities

All the synthesized compounds were evaluated for their cytotoxic activities *in vitro* against human cancer cell lines including chronic myeloid leukemia cell line K562, hepatocellular carcinoma cell line SMMC-7721, lung cancer cell line A549, human prostate cancer cell line PC-3 and human epidermoid tumor cell line KB under normoxic and hypoxic conditions. TPZ was employed as a positive control. The results are summarized in Table 1.

As shown in Table 1, some tested benzotriazine 1,4-dioxide derivatives showed higher or similar cytotoxic activity and selectivity in comparison with TPZ against the tested cancer cell lines except KB cell line. The substituent on the 3-position or 7-position of benzotriazine ring had significant impact on activity because of the disparity on the electronic and lipophilic properties of the molecule. Obviously, compounds (e.g. **4i**–**4n**) with an aromatic group at 3-position side chain terminal displayed more potent cytotoxic activity than those with a 3-alkyl side chain (e.g. **4a**–**4h**) against most tested cell lines. On the other hand, introduction of methoxy group into 7-position of benzotriazine ring improved both cytotoxicity and selectivity. For example, the cytotoxic activity and selectivity in hypoxia against K562 cell line of **4q** was 7.5 fold and 114 fold better than those of TPZ, respectively.

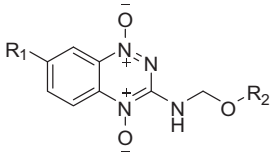
Comparing the cytotoxic activity of **4i** and **4k** with that of **4j**, **4m** and **4n** suggested that an electron-withdrawing group on benzene ring of 3-position side chain increased cytotoxicity against most tested cell lines, particularly for SMMC-7721 and K562. These 3-(alkoxymethylamino)-1,2,4- benzotriazine 1,4-dioxide compounds with oxygen atom at the 3-position side chain shown more favorable hypoxic selectivity compared with previous reported 3-amino-1,2,4-benzotriazine 1,4-dioxide compounds [26,27].

#### 2.2.2. Mechanism studies

To gain preliminary insight into the mechanisms of action of these compounds, **4b**, **4l** and **4m** were further assayed for their effect on cell cycle progression and proteins expression. The results are presented as follows.

DNA fragmentation is a common feature of apoptotic cell death. Propidium iodide staining for Sub-G1 content analysis is used to characterize the apoptosis process. As shown in Fig. 1(A), after

**Table 1**
Cytotoxicity of TPZ derivatives against five cancer cell lines in hypoxia and in normoxia.



| Compound | $R_1$ | $R_2$ | Cytotoxicity ($IC_{50}$, μM) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | SMMC-7721 | | K562 | | KB | | A549 | | PC3 | |
| | | | H[a] | N[b] | H | N | H | N | H | N | H | N |
| TPZ | H | – | 4.75 | 32.79 | 1.81 | 19.41 | 18.71 | 6.29 | 1.93 | 7.43 | 1.17 | 20.97 |
| **4a** | H | $CH_3$ | 2.48 | 6.67 | 3.79 | 7.8 | 9.07 | 1.58 | 2.77 | 7.45 | 4.27 | 7.51 |
| **4b** | H | $C_2H_5$ | 4.23 | 27.12 | 4.99 | 15.42 | 22.54 | 2.80 | 4.09 | 10.52 | 7.4 | 15.9 |
| **4c** | H | *n*-$C_3H_7$ | 5.53 | 19.52 | 3.89 | 17.61 | 10.21 | 1.95 | 3.74 | 7.46 | 7.4 | 14.2 |
| **4d** | H | *n*-$C_4H_9$ | 3.99 | 21.09 | 3.24 | 17.63 | 15.24 | 1.75 | 2.32 | 6.31 | 6.01 | 14.67 |
| **4e** | H | *n*-$C_5H_{11}$ | 3.05 | 16.98 | 3.3 | 14.53 | 39.54 | 1.22 | 2.90 | 6.46 | 8.51 | 9.2 |
| **4f** | H | *iso*-$C_5H_{11}$ | 2.86 | 15.87 | 5.85 | 13.11 | 5.21 | 3.04 | 2.11 | 7.27 | 7.52 | 11.06 |
| **4g** | H | Cyclohexyl | 4.17 | 28.15 | 4.88 | 10.78 | 22.08 | 4.39 | 1.20 | 5.18 | 6.67 | 3.25 |
| **4h** | H | *n*-$C_7H_{15}$ | 3.09 | 7.03 | 6.17 | 2.97 | 13.99 | 2.52 | 3.49 | 2.61 | 0.39 | 1.21 |
| **4i** | H | $CH_2$-$C_6H_5$ | 4.79 | 20.12 | 4.69 | 16.11 | 12.26 | 5.72 | 4.14 | 10.75 | 11.86 | 13.41 |
| **4j** | H | $CH_2$-$C_6H_4$-4-Cl | 0.81 | 14.5 | 1.85 | 3.26 | 8.26 | 1.30 | 2.19 | 1.47 | 5.1 | 5.76 |
| **4k** | H | $CH(CH_3)$-$C_6H_5$ | 2.47 | 12.9 | 2.24 | 8.72 | 14.01 | 2.51 | 2.81 | 2.71 | 10.67 | 6.08 |
| **4l** | H | $CH_2$-$C_6H_4$-4-OMe | 1.15 | 21.96 | 0.66 | 20.1 | 14.21 | 4.06 | 3.32 | 7.64 | 52.76 | 14.49 |
| **4m** | H | $CH_2$-$C_6H_4$-4-$CF_3$ | 0.78 | 9.45 | 1.24 | 18.3 | 19.8 | 0.39 | 4.27 | 8.42 | 9.31 | 7.65 |
| **4n** | H | $CH_2$-$C_6H_4$-3-$NO_2$ | 0.52 | 8.54 | 0.65 | 5.65 | 16.6 | 0.58 | 3.44 | 2.89 | >100 | 3.07 |
| **4o** | OMe | *n*-$C_3H_7$ | 1.07 | 2.88 | 3.52 | 4.28 | 11.99 | 3.20 | 2.54 | 2.81 | 10.61 | 6.57 |
| **4p** | OMe | *n*-$C_5H_{11}$ | 0.55 | 12.86 | 0.45 | 7.11 | 16.95 | 2.81 | 2.51 | 5.04 | 30.9 | 3.63 |
| **4q** | OMe | $CH_2$-$C_6H_5$ | 1.19 | 21.39 | 0.24 | 27.52 | 18.12 | 2.40 | 4.47 | 2.50 | 88.52 | 8.58 |
| **4r** | OMe | $CH(CH_3)$-$C_6H_5$ | 0.74 | 8.04 | 0.25 | 6.69 | 12.41 | 0.22 | 0.22 | 3.13 | >100 | 4.14 |

[a] H = hypoxia: the percentage of oxygen is 3%.
[b] N = normoxia: the percentage of oxygen is 20%.

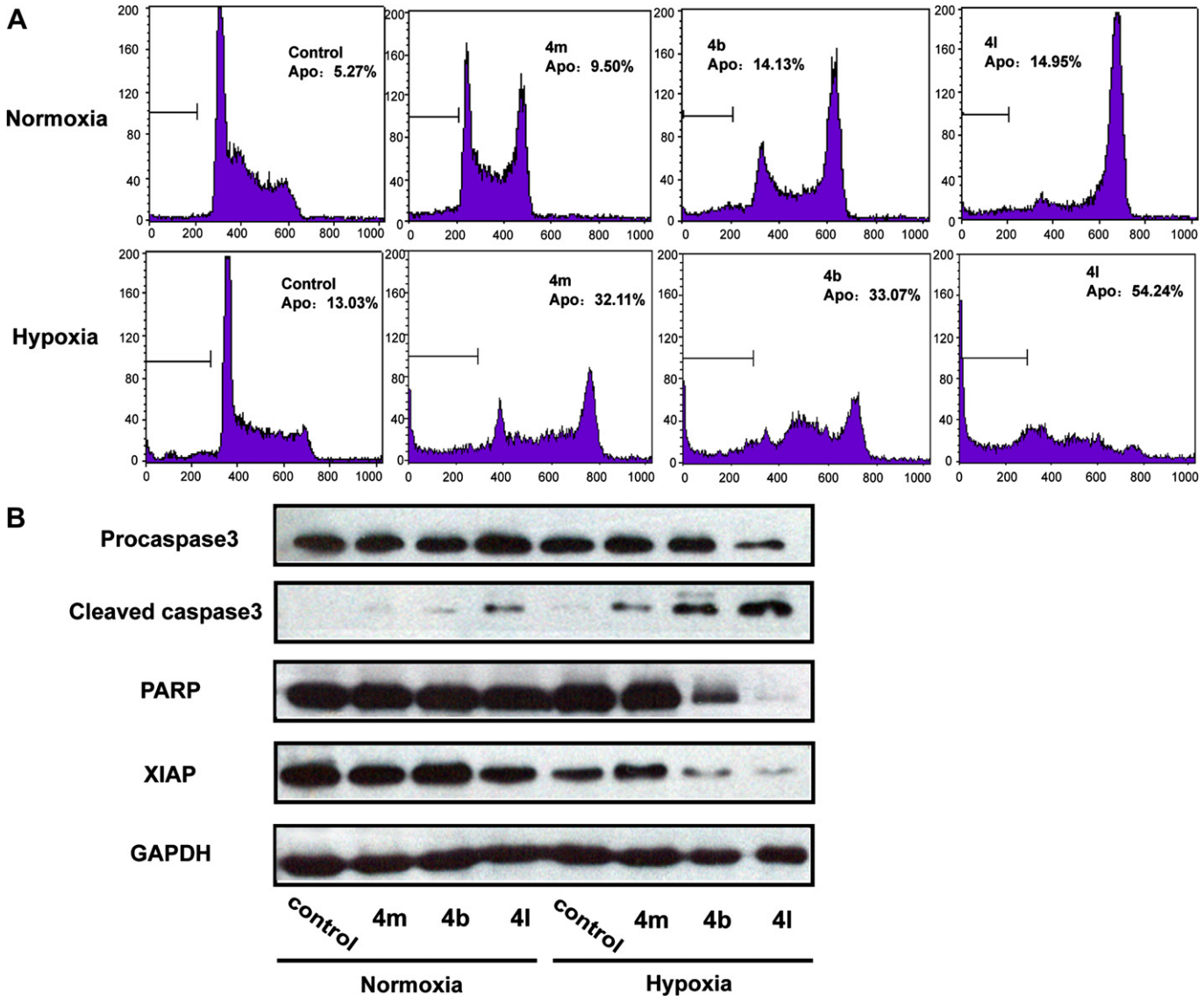



**Fig. 1.** (A) K562 cells were incubated in normoxia and in hypoxia, and were treated with **4m**, **4b**, **4l** (10 μM) for 48 h. After treatment, cells were harvested and detected of apoptosis by flow cytometry using PI apoptosis detection kit. (B) K562 cells were harvested after the same treatment as A, cell extract were collected and immunoblotted with procaspase-3, cleaved caspase-3, PARP and XIAP antibodies.

treatment with **4b**, **4l**, **4m** (10.0 μM) for 48 h in normoxia and in hypoxia, a characteristic cleavage of DNA was observed in K562 cells. The percentages of apoptotic cells induced by **4m**, **4b** and **4l** were 9.50%, 14.13% and 14.95% in normoxia, respectively, while that in hypoxia were 32.11%, 33.07% and 54.24%, respectively. Spontaneous apoptosis of the control was seen in 5.27% of cells in normoxia and 13.03% in hypoxia. These results suggested that these compounds serve as potential hypoxic-selective compounds, mainly owing to the undergoing of apoptosis.

In most cases, the activation of caspase cascade accompanies with the apoptosis. The expression of procaspase-3, cleaved caspase-3, XIAP and PARP were measured in K562 cells treated with compound **4m, 4b, 4l** (10.0 μM, 48 h). As shown in Fig. 1(B), **4m**, **4b** and **4l** decrease the protein levels of procaspase-3, XIAP and PARP, and induce the cleavage of caspase-3 in hypoxia.

### 2.3. Physico-chemical characteristics

Lipophilicity of the drug molecules plays an important role in relation to their biological activity. Baker et al. reported that the retention behavior of chemical substances in HPLC could be used in QSAR studies to replace the hydrophobicity parameter of drugs [29].

In order to investigate the relationship between lipophilicity and hypoxic activity, retention times of the 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives was measured by an RP-HPLC. And RI values of tested compounds were calculated by an equation

**Table 2**
Retention index (RI) of tirapazamine derivatives and their cytotoxicity against K562 cell line.

| Compound | RI | $IC_{50}$ (K562) | HCR | Compound. | RI | $IC_{50}$ (K562) | HCR |
|---|---|---|---|---|---|---|---|
| **4a** | 392.4 | 3.79 | 2.06 | **4j** | 737.8 | 1.85 | 1.76 |
| **4b** | 432.4 | 4.99 | 3.09 | **4k** | 720.5 | 2.24 | 3.89 |
| **4c** | 440.8 | 3.89 | 4.53 | **4l** | 603.7 | 0.66 | 30.45 |
| **4d** | 481.2 | 3.24 | 5.44 | **4m** | 632.3 | 1.24 | 16.21 |
| **4e** | 690.5 | 3.3 | 4.40 | **4n** | 658.1 | 0.65 | 8.69 |
| **4f** | 740.6 | 5.85 | 2.24 | **4o** | 430.2 | 3.52 | 1.22 |
| **4g** | 738.3 | 4.88 | 2.21 | **4p** | 647.9 | 0.45 | 15.80 |
| **4h** | 760.1 | 6.17 | 0.48 | **4q** | 585.2 | 0.24 | 114.67 |
| **4i** | 716.9 | 4.69 | 3.43 | **4r** | 628.4 | 0.25 | 26.76 |

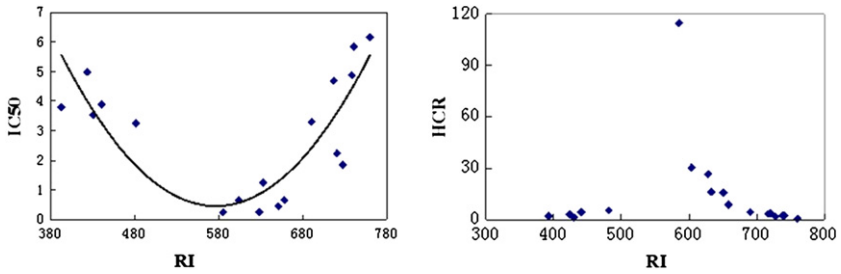



**Fig. 2.** The correlation between cytotoxicity or HCR of tirapazamine derivatives and their RI values.

based on the retention times of the alkan-2-ones used. The results are summarized in Table 2 and Fig. 2.

As shown in Fig. 2, hypoxic activities increased with the lipophilicity of compounds in the range from 380 to 580 in terms of RI values while negative correlation was shown when the RI values exceeded 580, indicating that introducing appropriate lipophilic groups enhanced cytotoxicity. On the other hand, compounds with the RI values in the range from 580 to 650 showed better hypoxic selectivity.

## 3. Conclusions

In summary, a new series of 3-(alkoxymethylamino)-1,2,4-benzotriazine 1,4-dioxides **4a**–**r** have been synthesized and evaluated for their hypoxic cytotoxicity and selectivity. Some compounds showed higher or similar cytotoxic activity compared to TPZ in hypoxia, particularly for SMMC-7721 and K562. Substituents on the benzotriazine ring or at the 3-position affected the activity, as introduction of a methoxy group at 7-position improved both cytotoxicity and selectivity. And compounds with aromatic group at 3-position side chain terminal were more active than corresponding compounds with alkyl groups. Physico-chemical study showed the positive correlation between hypoxic activity and lipophilicity within a certain range. Mechanism study using the potent derivatives **4b**, **4l** and **4m** revealed that the cytotoxic activities of these compounds might be mediated by inducing apoptosis.

## 4. Experimental protocols

### 4.1. Synthesis

General: melting points were obtained on a B-540 Büchi melting-point apparatus and are uncorrected. IR spectra were performed on a Brüker VECTOR 22 FTIR spectrophotometer in KBr pellets (400–4000 $cm^{-1}$). $^1H$ NMR spectra were recorded on a Brüker AM 400 instrument at 400 MHz (chemical shifts are expressed as $\delta$ values relative to TMS as internal standard). Mass spectra (MS), ESI (positive) were recorded on an Esquire-LC-00075 spectrometer.

#### 4.1.1. General procedure for the synthesis of 3-(Alkoxymethylamino)-1,2,4-benzotriazine 1-oxides *3a*–*r*

A mixture of substituted 3-amino-1,2,4-benzotriazine 1-oxides **2a**–**b** (1.1 mmol), paraformaldehyde (4.7 mmol) and corresponding alcohols (4 mL) was stirred at 85 °C for 3 h in the presence of catalytic amount of acetic acid. Then the solvent was removed under vacuum and the residue was purified by silica gel column chromatography (EtOAc/petrolemn ether, 1:2) to give pure compounds **3a**–**r**.

#### 4.1.2. 3-(Methoxymethylamino)-1,2,4-benzotriazine 1-oxide (*3a*)

Yellow solid (70.6% yield); IR (KBr, $cm^{-1}$) 3323, 3059, 2938, 2827, 1562, 1497, 1420, 1112, 1060, 902, 761, 643, 578; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.30 (d, 1H, $J = 8.0$ Hz, H-8), 7.75 (td, 1H, $J = 8.0$, 1.6 Hz, H-6), 7.69 (d, 1H, $J = 8.0$ Hz, H-5), 7.37 (td, 1H, $J = 8.0$, 1.6 Hz, H-7), 6.08 (br s, 1H, N–H), 5.03 (d, 2H, $J = 7.6$ Hz, $CH_2$), 3.44 (s, 3H, $CH_3$); MS (ESI): $m/s = 207$ $[M + 1]^+$.

#### 4.1.3. 3-(Ethoxymethylamino)-1,2,4-benzotriazine 1-oxide (*3b*)

Yellow solid (74.4% yield); IR (KBr, $cm^{-1}$) 3309, 3059, 2970, 2915, 1567, 1495, 1406, 1316, 1064, 1006, 763, 645; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.28 (d, 1H, $J = 8.0$ Hz, H-8), 7.74 (td, 1H, $J = 8.0$, 1.6 Hz, H-6), 7.67 (d, 1H, $J = 8.0$ Hz, H-5), 7.35 (td, 1H, $J = 8.0$, 1.6 Hz, H-7), 6.17 (br s, 1H, N–H), 5.07 (d, 2H, $J = 7.2$ Hz, $CH_2$), 3.65 (q, 2H, $J = 6.8$ Hz, $CH_2$), 1.24 (t, 3H, $J = 6.8$ Hz, $CH_3$); MS (ESI): $m/s = 221$ $[M + 1]^+$.

#### 4.1.4. 3-(Propoxymethylamino)-1,2,4-benzotriazine 1-oxide (*3c*)

Yellow solid (73.8% yield); IR (KBr, $cm^{-1}$) 3314, 3060, 2928, 2871, 1697, 1565, 1493, 1411, 1317, 1069, 941, 758, 658, 591; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.28 (d, 1H, $J = 8.8$ Hz, H-8), 7.74 (t, 1H, $J = 7.6$ Hz, H-6), 7.67 (d, 1H, $J = 8.0$ Hz, H-5), 7.36 (t, 1H, $J = 7.8$ Hz, H-7), 6.36 (br s, 1H, N–H), 5.07 (d, 2H, $J = 6.8$ Hz, $CH_2$), 3.54 (t, 2H, $J = 6.6$ Hz, $CH_2$), 1.63 (m, 2H, $CH_2$), 0.91 (t, 3H, $J = 7.4$ Hz, $CH_3$); MS (ESI): $m/s = 235$ $[M + 1]^+$.

#### 4.1.5. 3-(Butoxymethylamino)-1,2,4-benzotriazine 1-oxide (*3d*)

Yellow solid (88.0% yield); IR (KBr, $cm^{-1}$) 3311, 3063, 2945, 2865, 1564, 1491, 1415, 1357, 1237, 1074, 757, 644; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.28 (d, 1H, $J = 8.0$ Hz, H-8), 7.73 (t, 1H, $J = 8.0$ Hz, H-6), 7.67 (d, 1H, $J = 8.0$ Hz, H-5), 7.35 (t, 1H, $J = 8.0$ Hz, H-7), 6.38 (br s, 1H, N–H), 5.07 (d, 2H, $J = 7.2$ Hz, $CH_2$), 3.58 (t, 2H, $J = 6.8$ Hz, $CH_2$), 1.57 (m, 2H, $CH_2$), 1.35 (m, 2H, $CH_2$), 0.89 (t, 3H, $J = 7.2$ Hz, $CH_3$); MS (ESI): $m/s = 249$ $[M + 1]^+$.

#### 4.1.6. 3-(Pentyloxymethylamino)-1,2,4-benzotriazine 1-oxide (*3e*)

Yellow solid (79.8% yield); IR (KBr, $cm^{-1}$) 3304, 3060, 2925, 2860, 1563, 1492, 1413, 1319, 1236, 1075, 760, 647; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.27 (d, 1H, $J = 8.0$ Hz, H-8), 7.72 (t, 1H, $J = 7.6$ Hz, H-6), 7.66 (d, 1H, $J = 8.0$ Hz, H-5), 7.34 (t, 1H, $J = 7.8$ Hz, H-7), 6.29 (t, 1H, $J = 6.8$ Hz, N–H), 5.07 (d, 2H, $J = 6.8$ Hz, $CH_2$), 3.57 (t, 2H, $J = 6.6$ Hz, $CH_2$), 1.59 (m, 2H, $CH_2$), 1.29 (m, 4H, $CH_2$ and $CH_2$), 0.86 (t, 3H, $J = 6.8$ Hz, $CH_3$); MS (ESI): $m/s = 263$ $[M + 1]^+$.

#### 4.1.7. 3-(iso-Pentyloxymethylamino)-1,2,4-benzotriazine 1-oxide (*3f*)

Yellow solid (83.3% yield); IR (KBr, $cm^{-1}$) 3307, 3060, 2917, 2866, 1565, 1492, 1413, 1358, 1319, 1237, 1076, 763, 659; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.29 (d, 1H, $J = 8.0$ Hz, H-8), 7.74 (t, 1H, $J = 7.6$ Hz, H-6), 7.69 (d, 1H, $J = 8.0$ Hz, H-5), 7.36 (t, 1H, $J = 7.8$ Hz, H-7), 5.93 (t, 1H, $J = 6.8$ Hz, N–H), 5.06 (d, 2H, $J = 7.2$ Hz, $CH_2$), 3.61 (t, 2H, $J = 6.8$ Hz, $CH_2$), 1.69 (m, 1H, CH), 1.49 (m, 2H, $CH_2$), 0.88 (d, 6H, $J = 6.8$ Hz, $CH_3$ and $CH_3$); MS (ESI): $m/s = 263$ $[M + 1]^+$.

#### 4.1.8. 3-(Cyclohexyloxymethylamino)-1,2,4-benzotriazine 1-oxide (*3g*)

Yellow solid (76.3% yield); IR (KBr, $cm^{-1}$) 3308, 3056, 2926, 2851, 1564, 1496, 1410, 1313, 1240, 1061, 763, 661; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.28 (d, 1H, $J = 8.8$ Hz, H-8), 7.74 (t, 1H, $J = 7.6$ Hz, H-6), 7.66

(d, 1H, $J = 8.0$ Hz, H-5), 7.36 (t, 1H, $J = 8.0$ Hz, H-7), 6.02 (t, 1H, $J = 6.6$ Hz, N–H), 5.11 (d, 2H, $J = 7.2$ Hz, $CH_2$), 3.56 (m, 1H, CH), 1.95 (m, 2H, $CH_2$), 1.74 (m, 2H, $CH_2$), 1.35 (m, 6H, $CH_2$, $CH_2$ and $CH_2$); MS (ESI): $m/s = 275$ $[M + 1]^+$.

*4.1.9. 3-(Heptyloxymethylamino)-1,2,4-benzotriazine 1-oxide (**3h**)*

Yellow solid (76.2% yield); IR (KBr, $cm^{-1}$) 3304, 3060, 2923, 2854, 1563, 1493, 1414, 1360, 1320, 1238, 1098, 761, 657; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.28 (dd, 1H, $J = 8.6$, 1.2 Hz, H-8), 7.73 (td, 1H, $J = 7.6$, 1.6 Hz, H-6), 7.66 (d, 1H, $J = 7.6$ Hz, H-5), 7.35 (td, 1H, $J = 7.6$, 1.2 Hz, H-7), 6.11 (t, 1H, $J = 6.8$ Hz, N–H), 5.05 (d, 2H, $J = 7.2$ Hz, $CH_2$), 3.57 (t, 2H, $J = 6.4$ Hz, $CH_2$), 1.58 (m, 2H, $CH_2$), 1.29 (m, 8H, $4CH_2$), 0.86 (m, 3H, $CH_3$); MS (ESI): $m/s = 291$ $[M + 1]^+$.

*4.1.10. 3-(Benzyloxymethylamino)-1,2,4-benzotriazine 1-oxide (**3i**)*

Yellow solid (90.3% yield); IR (KBr, $cm^{-1}$) 3298, 3057, 2931, 2856, 1561, 1494, 1413, 1320, 1073, 955, 766, 725, 659, 653, 460; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.29 (d, 1H, $J = 8.0$ Hz, H-8), 7.75 (td, 1H, $J = 8.0$, 1.6 Hz, H-6), 7.69 (d, 1H, $J = 8.0$ Hz, H-5), 7.32 (m, 6H, H-7 and Ar), 6.19 (t, 1H, $J = 6.4$ Hz, N–H), 5.14 (d, 2H, $J = 7.6$ Hz, $CH_2$), 4.69 (d, 2H, $J = 8.8$ Hz, $CH_2$); MS (ESI): $m/s = 283$ $[M + 1]^+$.

*4.1.11. 3-((4-Chlorobenzyloxy)methylamino)-1,2,4-benzotriazine 1-oxide (**3j**)*

Yellow solid (77.7% yield); IR (KBr, $cm^{-1}$) 3320, 3053, 2926, 2857, 1696, 1564, 1493, 1413, 1318, 1075, 950, 794, 640, 586; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.29 (dd, 1H, $J = 8.0$, 0.8 Hz, H-8), 7.76 (td, 1H, $J = 8.0$, 1.6 Hz, H-6), 7.67 (d, 1H, $J = 8.0$ Hz, H-5), 7.38 (td, 1H, $J = 8.0$, 1.6 Hz, H-7), 7.31 (m, 4H, Ar), 6.14 (t, 1H, $J = 7.2$ Hz, N–H), 5.13 (d, 2H, $J = 7.6$ Hz, $CH_2$), 4.64 (s, 2H, $CH_2$); MS (ESI): $m/s = 317$ $[M + 1]^+$.

*4.1.12. 3-((1-Phenylethoxy)methylamino)-1,2,4-benzotriazine 1-oxide (**3k**)*

Yellow solid (79.8% yield); IR (KBr, $cm^{-1}$) 3319, 3063, 2985, 2929, 2865, 1567, 1492, 1419, 1375, 1318, 1238, 1041, 760, 685; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.26 (d, 1H, $J = 8.8$ Hz, H-8), 7.73 (td, 1H, $J = 7.6$, 0.8 Hz, H-6), 7.63 (d, 1H, $J = 8.8$ Hz, H-5), 7.30 (m, 6H, H-7 and Ar), 6.25 (t, 1H, $J = 7.0$ Hz, N–H), 4.99 (m, 2H, $CH_2$), 4.74 (q, 1H, $J = 6.5$ Hz, CH), 1.45 (d, 3H, $J = 6.8$ Hz, $CH_3$); MS (ESI): $m/s = 297$ $[M + 1]^+$.

*4.1.13. 3-((4-Methoxybenzyloxy)methylamino)-1,2,4-benzotriazine 1-oxide (**3l**)*

Yellow solid (81.6% yield); IR (KBr, $cm^{-1}$) 3332, 3027, 2930, 2868, 1561, 1505, 1413, 1317, 1245, 1071, 954, 814, 764, 644; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.29 (dd, 1H, $J = 8.0$, 0.8 Hz, H-8), 7.74 (td, 1H, $J = 8.0$, 1.6 Hz, H-6), 7.69 (d, 1H, $J = 8.0$ Hz, H-5), 7.37 (td, 1H, $J = 8.0$, 1.2 Hz, H-7), 7.29 (m, 2H, Ar), 6.88 (m, 2H, Ar), 6.06 (t, 1H, $J = 6.6$ Hz, N–H), 5.11 (d, 2H, $J = 6.8$ Hz, $CH_2$), 4.61 (d, 2H, $J = 5.6$ Hz, $CH_2$), 3.80 (d, 3H, $J = 6.8$ Hz, $CH_3$); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 159.50, 158.97, 148.77, 135.97, 131.63, 130.16, 129.76, 127.17, 126.07, 120.58, 114.02, 71.60, 70.22, 55.52; MS (ESI): $m/s = 313$ $[M + 1]^+$.

*4.1.14. 3-((4-(Trifluoromethyl)benzyloxy)methylamino)-1,2,4-benzotriazine 1-oxide (**3m**)*

Yellow solid (81.6% yield); IR (KBr, $cm^{-1}$) 3297, 3065, 2930, 2858, 1564, 1497, 1416, 1327, 1064, 1105, 1072, 953, 821, 763, 659; $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.75 (t, 1H, $J = 5.8$ Hz, N–H), 8.18 (d, 1H, $J = 8.4$ Hz, H-8), 7.85 (t, 1H, $J = 7.6$ Hz, H-5), 7.66 (m, 3H, H-6 and Ar), 7.56 (d, 2H, $J = 8.0$ Hz, Ar), 7.43 (t, 1H, $J = 7.8$ Hz, H-7), 4.96 (d, 2H, $J = 6.4$ Hz, $CH_2$), 4.69 (s, 2H, $CH_2$); MS (ESI): $m/s = 351$ $[M + 1]^+$.

*4.1.15. 3-((3-Nitrobenzyloxy)methylamino)-1,2,4-benzotriazine 1-oxide (**3n**)*

Yellow solid (81.6% yield); IR (KBr, $cm^{-1}$) 3324, 3091, 2941, 2835, 1564, 1527, 1412, 1347, 1121, 1065, 943, 809, 761, 664; $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.75 (t, 1H, $J = 6.4$ Hz, N–H), 8.17 (m, 2H, Ar), 8.10 (dd, 1H, $J = 8.0$, 1.2 Hz, H-8), 7.84 (td, 1H, $J = 8.0$, 1.2 Hz, H-6), 7.79 (d, 1H, $J = 8.0$ Hz, H-5), 7.62 (m, 2H, Ar), 7.43 (td, 1H, $J = 8.0$,1.6 Hz, H-7), 4.98 (d, 2H, $J = 6.8$ Hz, $CH_2$), 4.73 (s, 2H, $CH_2$); MS (ESI): $m/s = 328$ $[M + 1]^+$.

*4.1.16. 3-(Propoxymethylamino)-7-methoxy-1,2,4-benzotriazine 1-oxide (**3o**)*

Yellow solid (89.7% yield); IR (KBr, $cm^{-1}$) 3330, 3104, 2945, 2864, 1564, 1507, 1395, 1253, 1171, 1069, 1023, 844, 759, 646, 580; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.63 (m, 2H, H-8 and H-5), 7.45 (dd, 1H, $J = 8.8$, 2.4 Hz, H-6), 5.88 (t, 1H, $J = 6.8$ Hz, N–H), 5.07 (d, 2H, $J = 7.2$ Hz, $CH_2$), 3.95 (s, 3H, $CH_3$), 3.56 (t, 2H, $J = 6.8$ Hz, $CH_2$), 1.65 (m, 2H, $CH_2$), 0.94 (t, 3H, $J = 7.4$ Hz, $CH_3$); MS (ESI): $m/s = 265$ $[M + 1]^+$.

*4.1.17. 3-(Pentyloxymethylamino)-7-methoxy-1,2,4-benzotriazine 1-oxide (**3p**)*

Yellow solid (84.1% yield); IR (KBr, $cm^{-1}$) 3338, 2926, 2858, 1565, 1507, 1394, 1256, 1175, 1076, 1032, 942, 844, 758, 644, 568; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.60 (m, 2H, H-8 and H-5), 7.42 (dd, 1H, $J = 8.8$, 2.8 Hz, H-6), 6.02 (t, 1H, $J = 6.4$ Hz, N–H), 5.07 (d, 2H, $J = 6.8$ Hz, $CH_2$), 3.92 (s, 3H, $CH_3$), 3.56 (t, 2H, $J = 6.8$ Hz, $CH_2$), 1.59 (m, 2H, $CH_2$), 1.31 (m, 4H, CH2 and $CH_2$), 0.86 (t, 3H, $J = 7.0$ Hz, $CH_3$); MS (ESI): $m/s = 293$ $[M + 1]^+$.

*4.1.18. 3-(Benzyloxymethylamino)-7-methoxy-1,2,4-benzotriazine 1-oxide (**3q**)*

Yellow solid (84.5% yield); IR (KBr, $cm^{-1}$) 3311, 3054, 2935, 2848, 1568, 1504, 1393, 1310, 1264, 1124, 1058, 843, 735, 688, 575; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.60 (m, 2H, H-8 and H-5), 7.43 (dd, 1H, $J = 9.6$, 2.4 Hz, H-6), 7.32 (m, 5H, Ar–H), 5.88 (t, 1H, $J = 7.2$ Hz, N–H), 5.11 (d, 2H, $J = 7.2$ Hz, $CH_2$), 4.67(s, 2H, $CH_2$), 3.92 (s, 3H, $CH_3$); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 158.35, 158.04, 149.56, 145.06, 131.85, 129.40, 128.69, 128.45, 128.12, 120.10, 98.22, 71.99, 70.50, 56.38; MS (ESI): $m/s = 313$ $[M + 1]^+$.

*4.1.19. 3-((1-Phenylethoxy)methylamino)-7-methoxy-1,2,4-benzotriazine 1-oxide (**3r**)*

Yellow solid (83.7% yield); IR (KBr, $cm^{-1}$) 3308, 3050, 2933, 2846, 1571, 1502, 1379, 1305, 1257, 1121, 1053, 837, 729, 686, 561; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.58 (m, 2H, H-8 and H-5), 7.43 (dd, 1H, $J = 9.2$, 2.8 Hz, H-7), 7.31 (m, 5H, Ar), 5.81 (t, 1H, $J = 6.8$ Hz, N–H), 4.98 (m, 2H, $CH_2$), 4.73 (q, 1H, $J = 6.4$ Hz, CH), 3.93 (s, 3H, $CH_3$), 1.46 (d, 3H, $J = 6.4$ Hz, $CH_3$); MS (ESI): $m/s = 327$ $[M + 1]^+$.

*4.1.20. General procedure for the synthesis of 3-(Alkoxymethylamino)-1,2,4-benzotriazine 1,4-dioxides **4a**–**r***

A solution of MCPBA (0.30 mmol) in DCM (2 mL) was added dropwise to a stirred solution of 3-(alkoxymethylamino)-1,2,4-benzotriazine 1-oxides **3a**–**r** (0.22 mmol) in DCM (4 mL) and $NaHCO_3$ (0.44 mmol), and the mixture was stirred at room temperature for 6 h. The suspension was filtered through Celite, the solvent was evaporated, and the residue was purified by chromatography (EtOAc/DCM, 3:1), to give 3-(alkoxymethylamino)-1,2,4-benzotriazine 1,4-dioxides **4a**–**r** (about 50%) as a red solid, and the starting material **3a**–**r** (about 40%) which could be recycled.

*4.1.21. 3-(Methoxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4a**)*

Red solid (50.1% yield), mp 177–180 °C; IR (KBr, $cm^{-1}$) 3248, 3075, 2946, 2837, 1598, 1491, 1403, 1353, 1116, 1080, 951, 902, 857, 767, 660, 558; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.38 (m, 2H, H-8 and H-5), 7.93 (t, 1H, $J = 7.4$ Hz, H-6), 7.82 (br s, 1H, N–H), 7.60 (t, 1H, $J = 7.8$ Hz, H-7), 5.08 (d, 2H, $J = 5.6$ Hz, $CH_2$), 3.44 (s, 3H, $CH_3$); MS (ESI): $m/s = 223$ $[M + 1]^+$.

*4.1.22. 3-(Ethoxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4b**)*

Red solid (59.7% yield), mp 162–166 °C; IR (KBr, $cm^{-1}$) 3231, 3094, 2974, 2859, 1591, 1492, 1417, 1339, 1177, 1110, 1061, 948, 767, 653, 510; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 8.34 (m, 2H, H-8 and H-5), 7.91 (t, 1H, $J = 7.6$ Hz, H-6), 7.84 (br s, 1H, N–H), 7.57 (t, 1H, $J = 7.8$ Hz, H-7), 5.09 (d, 2H, $J = 6.0$ Hz, $CH_2$), 3.63 (q, 2H, $J = 7.2$ Hz, $CH_2$), 1.20 (t, 3H, $J = 7.2$ Hz, $CH_3$); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 149.92, 138.59, 136.17, 131.52, 128.17, 121.96, 117.99, 71.99, 64.51, 15.28; MS (ESI): $m/s = 237$ $[M + 1]^+$.

*4.1.23. 3-(Propoxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4c**)*

Red solid (58.2% yield), mp 160–163 °C; IR (KBr, $cm^{-1}$) 3243, 3100, 2926, 2862, 1595, 1492, 1416, 1175, 1076, 946, 765, 653, 510; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.34 (m, 2H, H-8 and H-5), 7.89 (m, 2H, H-6 and N–H), 7.55 (t, 1H, $J = 8.0$ Hz, H-7), 5.08 (d, 2H, $J = 5.6$ Hz, $CH_2$), 3.52 (t, 2H, $J = 6.8$ Hz, $CH_2$), 1.58 (m, 2H, $CH_2$), 0.89 (t, 3H, $J = 7.4$ Hz, $CH_3$); MS (ESI): $m/s = 251$ $[M + 1]^+$.

*4.1.24. 3-(Butoxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4d**)*

Red solid (58.2% yield), mp 158–160 °C; IR (KBr, $cm^{-1}$) 3254, 3027, 2949, 2860, 1598, 1492, 1413, 1350, 1175, 1079, 944, 860, 766, 721, 652; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.36 (m, 2H, H-8 and H-5), 7.91 (t, 1H, $J = 7.2$ Hz, H-6), 7.72 (br s, 1H, N–H), 7.58 (t, 1H, $J = 8.0$ Hz, H-7), 5.10 (d, 2H, $J = 5.6$ Hz, $CH_2$), 3.58 (t, 2H, $J = 6.6$ Hz, $CH_2$), 1.57 (m, 2H, $CH_2$), 1.36 (m, 2H, $CH_2$), 0.90 (t, 3H, $J = 7.4$ Hz, $CH_3$); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 149.93, 138.59, 136.19, 131.50, 128.14, 121.96, 117.98, 72.22, 68.91, 31.82, 19.47, 14.09; MS (ESI): $m/s = 265$ $[M + 1]^+$.

*4.1.25. 3-(Pentyloxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4e**)*

Red solid (62.2% yield), mp 133–135 °C; IR (KBr, $cm^{-1}$) 3254, 2955, 2932, 2857, 1594, 1493, 1418, 1361, 1181, 1111, 1081, 948, 864, 762, 721, 646; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.38 (m, 2H, H-8 and H-5), 7.93 (t, 1H, $J = 7.6$ Hz, H-6), 7.87 (br s, 1H, N–H), 7.59 (t, 1H, $J = 8.0$ Hz, H-7), 5.11 (d, 2H, $J = 5.6$ Hz, $CH_2$), 3.59 (t, 2H, $J = 6.4$ Hz, $CH_2$), 1.60 (m, 2H, $CH_2$), 1.32 (m, 4H, $CH_2$ and $CH_2$), 0.88 (t, 3H, $J = 7.4$ Hz, $CH_3$); MS (ESI): $m/s = 279$ $[M + 1]^+$.

*4.1.26. 3-(iso-Pentyloxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4f**)*

Red solid (63.9% yield), mp 153–156 °C; IR (KBr, $cm^{-1}$) 3250, 2950, 2933, 2864, 1593, 1493, 1417, 1360, 1335, 1181, 1166, 1109, 1077, 948, 764, 721, 659; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.37 (m, 2H, H-8 and H-5), 7.92 (t, 1H, $J = 7.2$ Hz, H-6), 7.79 (br s, 1H, N–H), 7.58 (t, 1H, $J = 8.0$ Hz, H-7), 5.10 (d, 2H, $J = 5.6$ Hz, $CH_2$), 3.61 (t, 2H, $J = 6.8$ Hz, $CH_2$), 1.67 (m, 2H, $CH_2$), 1.49 (m, 1H, CH), 0.89 (d, 6H, $J = 6.4$ Hz, $CH_3$ and $CH_3$); MS (ESI): $m/s = 279$ $[M + 1]^+$.

*4.1.27. 3-(Cyclohexyloxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4g**)*

Red solid (51.7% yield), mp 154–158 °C; IR (KBr, $cm^{-1}$) 3258, 2928, 2849, 1592, 1493, 1418, 1362, 1335, 1182, 1166, 1110, 1065, 949, 862, 763, 720, 646; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.34 (m, 2H, H-8 and H-5), 7.90 (t, 1H, $J = 7.8$ Hz, H-6), 7.80 (br s, 1H, N–H), 7.55 (t, 1H, $J = 8.0$ Hz, H-7), 5.13 (d, 2H, $J = 6.0$ Hz, $CH_2$), 3.54 (m, 1H, CH), 1.91 (m, 2H, $CH_2$), 1.72 (m, 2H, $CH_2$), 1.30 (m, 6H, $CH_2$, $CH_2$ and $CH_2$); MS (ESI): $m/s = 291$ $[M + 1]^+$.

*4.1.28. 3-(Heptyloxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4h**)*

Red solid (56.7% yield), mp 156–159 °C; IR (KBr, $cm^{-1}$) 3256, 2956, 2926, 2853, 1593, 1546, 1492, 1460, 1418, 1360, 1335, 1181, 1165, 1110, 1091, 1063, 947, 863, 761, 720, 645; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.35 (m, 2H, H-8 and H-5), 7.92 (t, 1H, $J = 7.6$ Hz, H-6), 7.88 (br s, 1H, N–H), 7.56 (t, 1H, $J = 8.0$ Hz, H-7), 5.09 (d, 2H, $J = 6.4$ Hz, $CH_2$), 3.56 (t, 2H, $J = 6.4$ Hz, $CH_2$), 1.56 (m, 2H, $CH_2$), 1.28 (m, 8H, $4CH_2$), 0.85 (m, 3H, $CH_3$); MS (ESI): $m/s = 307$ $[M + 1]^+$.

*4.1.29. 3-(Benzyloxymethylamino)-1,2,4-benzotriazine 1,4-dioxide (**4i**)*

Red solid (53.4% yield), mp 158–162 °C; IR (KBr, $cm^{-1}$) 3342, 2925, 2857, 1601, 1491, 1409, 1347, 1172, 1079, 962, 854, 723; $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.20 (t, 1H, $J = 6.8$ Hz, N–H), 8.24 (d, 1H, $J = 9.2$ Hz, H-8), 8.19 (d, 1H, $J = 9.2$ Hz, H-5), 7.97 (td, 1H, $J = 8.0$, 1.2 Hz, H-6), 7.63 (td, 1H, $J = 8.0$, 0.8 Hz, H-7), 7.32 (m, 5H, Ar), 4.95 (d, 2H, $J = 6.4$ Hz, $CH_2$), 4.59 (s, 2H, $CH_2$); MS (ESI): $m/s = 299$ $[M + 1]^+$.

*4.1.30. 3-((4-Chlorobenzyloxy)methylamino)-1,2,4-benzotriazine 1,4-dioxide (**4j**)*

Red solid (49.3% yield), mp 196–198 °C; IR (KBr, $cm^{-1}$) 3234, 3100, 2932, 2821, 1595, 1490, 1413, 1341, 1175, 1077, 953, 859, 767, 654, 560; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.36 (m, 2H, H-8 and H-5), 7.93 (t, 1H, $J = 7.6$ Hz, H-6), 7.88 (br s, 1H, N–H), 7.59 (t, 1H, $J = 8.0$ Hz, H-7), 7.27 (s, 4H, Ar), 5.17 (d, 2H, $J = 6.4$ Hz, $CH_2$), 4.64 (s, 2H, $CH_2$); MS (ESI): $m/s = 333$ $[M + 1]^+$.

*4.1.31. 3-((1-Phenylethoxy)methylamino)-1,2,4-benzotriazine 1,4-dioxide (**4k**)*

Red solid (55.4% yield), mp 149–152 °C; IR (KBr, $cm^{-1}$) 3258, 3084, 2891, 1590, 1492, 1418, 1362, 1332, 1183, 1166, 1113, 1069, 1041, 952, 861, 764, 721, 700, 646; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.32 (m, 2H, H-8 and H-5), 7.89 (t, 1H, $J = 7.8$ Hz, H-6), 7.67 (br s, 1H, N–H), 7.56 (t, 1H, $J = 7.8$ Hz, H-7), 7.26 (m, 5H, Ar), 4.52 (m, 2H, $CH_2$), 4.72 (q, 1H, $J = 6.8$ Hz, CH), 1.45 (d, 3H, $J = 6.8$ Hz, $CH_3$); MS (ESI): $m/s = 313$ $[M + 1]^+$.

*4.1.32. 3-((4-Methoxybenzyloxy)methylamino)-1,2,4-benzotriazine 1-oxide 1,4-dioxide (**4l**)*

Red solid (52.8% yield), mp 174–177 °C; IR (KBr, $cm^{-1}$) 3236, 3103, 2924, 2876, 1597, 1504, 1414, 1344, 1245, 1175, 1115, 1064, 953, 859, 766, 720, 654, 527; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 8.34 (m, 2H, H-8 and H-5), 7.90 (m, 2H, H-6 and N–H), 7.57 (t, 1H, $J = 8.0$ Hz, H-7), 7.26 (d, 2H, $J = 8.0$ Hz, Ar), 6.83 (d, 2H, $J = 8.4$ Hz, Ar), 5.14 (d, 2H, $J = 7.0$ Hz, $CH_2$), 4.59 (s, 2H, $CH_2$), 3.75 (s, 3H, $CH_3$); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 159.59, 149.87, 138.56, 136.16, 131.54, 129.68, 129.45, 128.20, 121.93, 117.98, 114.08, 71.41, 70.65, 55.49; MS (ESI): $m/s = 329$ $[M + 1]^+$.

*4.1.33. 3-((4-(Trifluoromethyl)benzyloxy)methylamino)-1,2,4-benzotriazine 1,4-dioxide (**4m**)*

Red solid (45.9% yield), mp 190–193 °C; IR (KBr, $cm^{-1}$) 3257, 3107, 2931, 1594, 1494, 1416, 1359, 1333, 1184, 1163, 1112, 1067, 1018, 952, 866, 824, 765, 722, 660; $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.19 (t, 1H, $J = 7.0$ Hz, N–H), 8.24 (d, 1H, $J = 8.4$ Hz, H-8), 8.18 (d, 1H, $J = 8.4$ Hz, H-6), 7.98 (t, 1H, $J = 8.0$ Hz, H-5), 7.65 (m, 3H, H-7 and Ar), 7.55 (d, 2H, $J = 8.4$ Hz, Ar), 4.98 (d, 2H, $J = 6.8$ Hz, $CH_2$), 4.70 (s, 2H, $CH_2$); $^{13}C$ NMR (125 MHz, DMSO-$d_6$) $\delta$ 150.27, 143.99, 143.46, 139.15, 136.30, 131.63, 128.64, 128.54, 125.71, 125.69, 121.84, 117.96, 71.84, 68.96; MS (ESI): $m/s = 367$ $[M + 1]^+$.

*4.1.34. 3-((3-Nitrobenzyloxy)methylamino)-1,2,4-benzotriazine 1,4-dioxide (**4n**)*

Red solid (35.8% yield), mp 186–189 °C; IR (KBr, $cm^{-1}$) 3241, 3101, 2932, 2825, 1593, 1527, 1415, 1350, 1176, 1077, 950, 732, 654, 555; $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.22 (t, 1H, $J = 6.4$ Hz, N–H), 8.24 (d, 1H, $J = 8.4$ Hz, H-8), 8.18 (m, 2H, H-5 and Ar), 8.09 (d, 1H,

$J = 8.4$ Hz, Ar), 7.98 (t, 1H, $J = 7.8$ Hz, H-6), 7.80 (d, 1H, $J = 7.2$ Hz, H-7), 7.63 (m, 2H, Ar), 5.00 (d, 2H, $J = 6.4$ Hz, $CH_2$), 4.74(s, 2H, $CH_2$); MS (ESI): $m/s = 344$ $[M + 1]^+$.

#### 4.1.35. 3-(Propoxymethylamino)-7-methoxy-1,2,4-benzotriazine 1,4-dioxide (**4o**)

Red solid (53.6% yield), mp 149–153 °C; IR (KBr, $cm^{-1}$) 3253, 3077, 2930, 2867, 1592, 1507, 1388, 1335, 1250, 1119, 1084, 969, 731, 633, 550; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.23 (d, 1H, $J = 9.6$ Hz, H-8), 7.73 (t, 1H, $J = 6.8$ Hz, N–H), 7.58 (d, 1H, $J = 2.4$ Hz, H-5), 7.51 (dd, 1H, $J = 9.6$, 2.8 Hz, H-6), 5.06 (d, 2H, $J = 6.8$ Hz, $CH_2$), 3.95 (s, 3H, $CH_3$), 3.52 (t, 2H, $J = 6.8$ Hz, $CH_2$), 1.58 (m, 2H, $CH_2$), 0.88 (t, 3H, $J = 7.2$ Hz, $CH_3$); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 159.71, 149.07, 134.62, 132.13, 129.49, 119.33, 99.31, 72.24, 70.69, 56.66, 23.04, 10.75; MS (ESI): $m/s = 281$ $[M + 1]^+$.

#### 4.1.36. 3-(Pentyloxymethylamino)-7-methoxy-1,2,4-benzotriazine 1,4-dioxide (**4p**)

Red solid (62.0% yield), mp 133–135 °C; IR (KBr, $cm^{-1}$) 3260, 3074, 2932, 2857, 1591, 1508, 1420, 1383, 1336, 1258, 1244, 1193, 1153, 1115, 1085, 1021, 973, 817, 797, 731, 629; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.24 (d, 1H, $J = 9.2$ Hz, H-8), 7.69 (t, 1H, $J = 6.6$ Hz, N–H), 7.60 (d, 1H, $J = 2.8$ Hz, H-5), 7.52 (dd, 1H, $J = 9.6$, 2.4 Hz, H-6), 5.06 (d, 2H, $J = 7.2$ Hz, $CH_2$), 3.95 (s, 3H, $CH_3$), 3.55 (t, 2H, $J = 6.6$ Hz, $CH_2$), 1.57 (m, 2H, $CH_2$), 1.29 (m, 4H, $CH_2$ and $CH_2$), 0.86 (t, 3H, $J = 7.0$ Hz, $CH_3$); MS (ESI): $m/s = 309$ $[M + 1]^+$.

#### 4.1.37. 3-(Benzyloxymethylamino)-7-methoxy-1,2,4-benzotriazine 1,4-dioxide (**4q**)

Red solid (48.6% yield), mp 148–152 °C; IR (KBr, $cm^{-1}$) 3256, 2939, 2821, 1604, 1504, 1383, 1328, 1259, 1112, 1015, 970, 835, 788, 691, 554; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.22 (d, 1H, $J = 9.2$ Hz, H-8), 7.76 (t, 1H, $J = 6.8$ Hz, N–H), 7.59 (d, 1H, $J = 2.4$ Hz, H-5), 7.52 (dd, 1H, $J = 9.2$, 2.4 Hz, H-6), 7.28 (m, 5H, Ar), 5.14 (d, 2H, $J = 6.8$ Hz, $CH_2$), 4.66 (s, 2H, $CH_2$), 3.95 (s, 3H, $CH_3$); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 159.82, 148.95, 137.50, 134.62, 132.26, 129.46, 128.70, 128.12, 128.02, 119.38, 99.30, 71.74, 70.92, 56.67; MS (ESI): $m/s = 329$ $[M + 1]^+$.

#### 4.1.38. 3-((1-Phenylethoxy)methylamino)-7-methoxy-1,2,4-benzotriazine 1,4-dioxide (**4r**)

Red solid (46.7% yield), mp 156–159 °C; IR (KBr, $cm^{-1}$) 3354, 3111, 2967, 2826, 1591, 1509, 1454, 1418, 1384, 1329, 1265, 1160, 1120, 1079, 1032, 1014, 956, 835, 793, 762, 728, 701, 614, 552; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.19 (d, $J = 9.6$ Hz, 1H, H-8), 7.59 (d, $J = 2.4$ Hz, 1H, H-5), 7.51 (m, 2H, H-6 and N–H), 7.30 (m, 4H, Ar), 7.18 (t, 1H, $J = 7.2$ Hz, Ar), 4.99 (m, 2H, $CH_2$), 4.71 (q, 1H, $J = 6.4$ Hz, CH), 3.96 (s, 3H, $CH_3$), 1.44 (d, 3H, $J = 6.4$ Hz, $CH_3$); MS (ESI): $m/s = 343$ $[M + 1]^+$.

### 4.2. Pharmacology

Five human cancer cell lines (K562, SMMC-7721, A549, PC-3, and KB) were purchased from Cell Bank of China Science Academy (Shanghai, China). The above cells were cultured in RPMI-1640 (Invitrogen Corp., Carlsbad, CA) medium with heat-inactivated 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) and incubated in hypoxic atmosphere with 3% $O_2$, 5% $CO_2$ and in normoxic atmosphere with 20% $O_2$, 5% $CO_2$ at 37 °C.

#### 4.2.1. Cytotoxicity assay [30]

The cytotoxic activity in vitro was measured using the MTT assay. All the compounds were dissolved in DMSO at the concentrations 10.0 mg/mL and were then diluted to the appropriate concentrations. Cells were plated in 96-well plates ($5 \times 10^3$ per well) for 24 h and subsequently treated with different concentrations of all tested compounds under normoxia or hypoxia for 72 h, respectively. Viable cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit (MTT, Sigma) according to the manufacturer's instructions. The concentration of drug causing 50% inhibition in absorbance compared with control cells ($IC_{50}$) was calculated using the software of dose–effect analysis with microcomputers.

#### 4.2.2. Flow cytometry analysis [30]

DNA content was monitored using flow cytometry. After drug exposure, K562 cells were harvested, washed with PBS, centrifuged, and fixed with 75% ethanol at −20 °C overnight. The fixed cells were resuspended in 500.0 μl of PBS containing 100 μg/ml RNase and incubated at 37 °C for 30 min. After incubation, the cells were stained with 10 μg/ml propidium iodide (PI, Sigma, St. Louis, MO) for 30 min at room temperature in the dark. Flow cytometry was analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

#### 4.2.3. Western blot analysis [30]

After treatment, cells were collected and lysed in a lysis buffer [150 mM NaCl, 50 mM Tris–HCl pH 8.0, 2 mM ethylene glycol-bis (b-aminoethyl ether), 2 mM EDTA, 25 mM NaF, 25 mM b-glycerophos-phate, 0.2% Triton X-100, 0.3% Nonidet P-40, and 0.1 mM phenylmethyl-sulfonyl fluoride]. Protein extracts were resolved by 8–15% SDS-PAGE and transferred to PVDF membranes, and western blot analysis was carried out using specific primary antibodies and then HRP-labeled secondary antibodies. The signals were visualized using enhanced chemiluminescent (ECL) detection reagents.

### 4.3. Retention index

#### 4.3.1. Chromatographic conditions

The analysis was performed on a Diamonsil $C_{18}$ column (4.6 mm 200 mm, 5 μm). The optimum separation in HPLC was carried out with a mobile phase composed of methanol:water (60:40, v/v) at a flow-rate of 1.0 ml/min. The volume of sample injected was 20 μl, the column temperature was set at 20 °C, and the isocratic elution condition was monitored at 280 or 350 nm, according to the UV absorption of the sample.

#### 4.3.2. RI evaluation

Each of the 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives and alkan-2-ones was dissolved in methanol (0.1 mg/ml) and then injected into the HPLC apparatus. The retention times of the 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives and alkan-2-ones were recorded. The capacity factors ($K_x'$) and RI values of the samples were calculated using the equations as follows:

$$K'_x = (t_x - t_0)/t_0$$

$$\mathrm{RI} = 100N + 100(\log K'_x - \log K'_n)/(\log K'_{n+1} - \log K'_n)$$

where $x$ = 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives measured, $n$ = alkan-2-one eluting immediately before $x$, $n + 1$ = alkan-2-one eluting immediately after $x$ and $N$ = carbon number of alkan-2-one. The $K'$ values were calculated using the retention time of $NaNO_2$ as a dead time.

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